Colonization, isolation, and cultural descriptions of *Thelephora terrestris* and other ectomycorrhizal fungi of shortleaf pine seedlings grown in fumigated soil

DONALD H. MARX AND W. CRAIG BRYAN
United States Department of Agriculture, Forest Service, Southeastern Forest Experiment Station, Athens, Georgia
AND
LARRY F. GRAND
Department of Plant Pathology, North Carolina State University, Raleigh, North Carolina
Received June 10, 1969

MARX, D. H., W. C. BRYAN, and L. F. GRAND. 1970. Colonization, isolation, and cultural descriptions of *Thelephora terrestris* and other ectomycorrhizal fungi of shortleaf pine seedlings grown in fumigated soil. Can. J. Bot. **48**: 207–211.

Nineteen fungal symbionts were isolated from ectomycorrhizae of 7- to 9-month-old shortleaf pine seedlings grown in heavily fumigated soil in a greenhouse. Cultural and chemical characterization revealed five distinct cultural groups. One symbiont group was culturally identical with isolates of *Thelephora terrestris*, a primary symbiont colonizer of fumigated soil. The other four groups of ectomycorrhizal fungi belonged to different species. Numerous surface sterilants were tested in attempts to isolate symbionts, but only mercuric chloride (100 p.p.m.) and copper sulfate (2.5 and 5%) were effective.

Introduction

Attempts at introducing pure cultures of ectomycorrhizal fungi[1] onto roots of various species of trees growing in sterile or partially sterile soil in open containers or nursery beds have been largely unsuccessful, primarily because of the rapid colonization of the soil by undesired ectomycorrhizal fungi. Noninoculated tree seedlings eventually have as many ectomycorrhizae as do inoculated seedlings (1, 4, 12).

In the southern United States, *Thelephora terrestris* (Ehrh.) Fr. is one of the primary ectomycorrhizal fungi which colonize fumigated or sterilized soils. Fruiting bodies of this symbiont have appeared rapidly in fumigated forest nurseries of slash pine (*Pinus elliottii* Engelm. var. *elliottii*), and hyphal strands have been traced to ectomycorrhizae of a single morphological form. Numerous cultures obtained by direct isolation from these ectomycorrhizae were morphologically identical, and their hyphal characteristics were similar to hyphae at the base of the sporophore (13). Fassi and Fontana (2) described the ectomycorrhizae formed by this symbiont on roots of *Pinus strobus* L. in Italian nurseries. Two greenhouse studies have been reported: in the first (8), *T. terrestris* colonized, fruited, and formed ectomycorrhizae with shortleaf pine (*P. echinata* Mill.); in the second (3), it colonized, fruited, and formed ectomycorrhizae with Virginia pine (*P. virginiana* Mill.). Seedlings in the first study were grown in pots of autoclaved soil; those in the second study were grown in pots of horticultural grade perlite. This fungus fruited abundantly and apparently formed ectomycorrhizae with pitch pine (*P. rigida* Mill.) in the nearly sterile wastes from anthracite mining in Pennsylvania (11). Recently, *T. terrestris* fruited and formed ectomycorrhizae with seven additional pine species which had been inoculated with pure cultures in flats in a special air-filtered, plant-growth room (7). Because of its wide range of pine hosts and its ability to colonize soil rapidly, *T. terrestris* is probably the major ectomycorrhizal associate of pine outplants in the southern region and possibly in other regions of the United States.

More insight into the nature of pine ectomycorrhizal associations formed after soil fumigation is required for long-range field experimentation on ectomycorrhizae. The purpose of the study reported here was (*a*) to determine the different ectomycorrhizal symbionts of shortleaf pine that colonize heavily fumigated soil, (*b*) to devise better methods of direct isolation of symbiotic fungi from ectomycorrhizae, and (*c*) to describe all isolates and culturally compare them with known isolates of *T. terrestris*.

[1]Terminology suggested by Peyronel *et al.* (10).

Materials and Methods

Shortleaf pine seed were washed and stratified in moist vermiculite at 5 °C for 2 weeks and planted in fumigated flats of soil (2 parts sandy loam, 1 part washed sand, and 1 part peat moss, v/v/v). The flats were placed in a partially shaded greenhouse (65 klux light) and watered on alternate days. After 3 months, the seedlings were removed and planted individually in unused 10-cm clay pots of soil. The soil had previously been fumigated twice at the rate of 0.45 kg of methyl bromide – chloropicrin per 0.5 cubic meter. After 4 months of growth in the greenhouse, 12 to 15 seedlings were removed every 2 weeks for 3 months, the soil was washed from the roots, and lateral root segments with representative ectomycorrhizae were excised. Ectomycorrhizae on 2- to 3-cm lengths of lateral roots were examined at 4× magnification, and all adhering soil particles were removed. Root segments were placed in perforated plastic vials, washed vigorously in 1800 ml of detergent for 1 min, and placed without rinsing in 1800 ml of surface sterilant for varying periods of time. The vials were removed from the sterilant and vigorously washed for 15 to 20 min in three changes each of 1800 ml of sterile distilled water. After they had been left overnight at 5 °C in a fourth volume of sterile water, the vials were soaked briefly in a final volume. The ectomycorrhizae were then removed aseptically from the vials, excised from lateral roots, placed singly in tubes of modified Melin-Norkrans (MMN) agar medium (6), and incubated at 23 °C in diffuse light for 8 weeks.

The following surface sterilants and exposures were tested: (*a*) mercuric chloride at 100 parts per million (p.p.m.) with and without 1% zepherin chloride for 1.5, 2.0, 2.5, or 3.0 min; (*b*) mercuric chloride at 100 p.p.m. for 2.5 min preceded by a 30-s wash in 70% ethyl alcohol or followed by 15 or 30 min in 1% streptomycin sulfate; (*c*) chlorohexidine acetate at 5000 p.p.m. for 10, 20, or 30 min with or without 1% zepherin chloride; (*d*) hydrated copper sulfate at 1.0, 2.5 or 5.0% for 10, 20, or 30 min with or without a 30-s prewash in 70% ethyl alcohol; (*e*) formaldehyde at 1.0, 2.5, 5.0, or 7.5% for 1.0, 2.5, or 5.0 min with or without a post-soak for 15 or 30 min in 1% streptomycin sulfate; (*f*) 1% potassium dichromate, silver nitrate, or chromium potassium sulfate for 1 or 10 min; and (*g*) phenol at 1.0, 2.5, or 5.0% for 1 or 10 min. Twenty ectomycorrhizae were used for each sterilant–exposure combination. Isolated fungal cultures with clamp connections were considered to be ectomycorrhizal symbionts.

Cultures of *Thelephora terrestris* used in characterization studies were isolates 1 and 2 obtained from shortleaf pine ectomycorrhizae (8); isolate S-142 cultured from a sporophore by B. Zak, Forestry Sciences Laboratory, Corvallis, Oregon; isolate Z-13 obtained from an ectomycorrhiza by B. Zak; isolate V-9037 obtained from a sporophore rhizomorph by O. Vaartaja, Forest Research Laboratory, Maple, Ontario, Canada; and isolate OKM-3838, a polysporous culture obtained from O. K. Miller, Jr., Forest Tree Disease Laboratory, Laurel, Maryland. All cultures were maintained on MMN agar medium for stock usage.

Isolates were grown at 25 °C in darkness on 30 ml of Hagem (9), MMN, and Difco potato–dextrose agar (PDA) in Petri dishes. Each isolate was replicated 8 times. Radial growth measurements and culture descriptions were made after 25 days. Ridgway's color standards and nomenclature were used to describe colony color. Hyphae were mounted in lactophenol with or without cotton blue or phloxine and examined microscopically by transmitted and phase lighting. Mycelial reaction to 5% KOH and 10% NH_4OH was also noted.

Symbiont antagonism toward *Phytophthora cinnamomi* Rands, *P. drechsleri* Tucker, *P. cactorum* (Lebert & Cohn) Schroeter, *P. citricola* Sawada, *P. heveae* Thompson, *Pythium aphanidermatum* (Edson) Fitz., *P. irregulare* Buisman, *P. spinosum* Sarvada, and *P. vexans* deBary was also tested on MMN agar medium. After 20 days, inoculum of the test pathogens was placed 2 cm from the mycelial margin of the symbiont (6). The widths of the inhibition zones were measured after 5 days at 25 °C for the *Phytophthora* species and after 3 days at 25 °C for the *Pythium* species.

Results

Sporophores of *Thelephora terrestris* developed around the stems of shortleaf pine seedlings during the fourth and fifth months and persisted for several weeks. An unreported and unusual sporophore formation was observed on the outside surfaces of about half of the clay pots. The sporophore, which ranged from 1 to 6 cm in width, was resupinate and fanshaped. Its hymenial surface was paper-thin but released thousands of basidiospores per square centimeter when incubated overnight over plates of water agar. The basidiospores did not germinate. Apparently, *T. terrestris* grew through the pores (from 8 to 12 μ in diameter) of the clay pots. Hyphal fragments with typical clamp connections were observed in pieces of crushed clay removed from the pots at points under the sporophores. Numerous ectomycorrhizal clusters were found in the root substrate immediately adjacent to the externally formed sporophores.

An unidentified *Marasmius* species also fruited in several pots during the seventh and eighth months of the study. Basidiospores of this fungus were collected but did not germinate.

All shortleaf pine seedlings had excellent ectomycorrhizal development. From 70 to 85% of all short roots were mycorrhizal as early as the fifth month of growth. One morphological form of ectomycorrhizae was prevalent during the fifth through the sixth month. This form was white to cream colored, bifurcate to coralloid, and was identical with that previously reported for *T. terrestris* on aseptic roots of shortleaf pine (8). Another form was observed during the seventh month. This form was less abundant

than the above and was tan to dark brown and primarily complex coralloid.

Nineteen fungal symbiont cultures were obtained from platings of 1350 ectomycorrhizae. These isolates were designated with the symbol MST. Symbiont recovery was 10% with mercuric chloride without additives or additional treatment and 5% with 2.5 or 5.0% copper sulfate without additives or additional treatments. Phenol, 1% copper sulfate, formaldehyde, potassium dichromate, silver nitrate, and chromium potassium sulfate were unsuccessful. Although symbionts were not recovered, the selective effects of these latter chemicals on surface microflora of ectomycorrhizae are interesting. Formaldehyde in all combinations eliminated completely all fungal contaminants but had little or no effect on surface bacteria. Silver nitrate eliminated all microorganisms. Ectomycorrhizae exposed to the other test chemicals yielded about half fungal and half bacterial contaminants without any aseptic ectomycorrhizae after 3 weeks' incubation. Streptomycin sulfate in combination with other chemicals did not significantly reduce bacterial contamination. Similarly, prewash for 30 s in 70% ethyl alcohol did not reduce contamination. Zepherin chloride, a widely used antiseptic in medicine, actually increased bacterial contamination and inhibited recovery of symbionts from ectomycorrhizae.

Fourteen of the 19 MST-isolates were used in characterization studies. The symbionts used in these studies and the surface sterilants used for their isolation were MST-6, 12, 13, and 14 (2.5 min in 100 p.p.m. mercuric chloride); MST-7 (1.5 min in 100 p.p.m. mercuric chloride); MST-8 and 11 (3 min in 100 p.p.m. mercuric chloride); MST-9 and 10 (2 min in 100 p.p.m. mercuric chloride); MST-15 and 17 (20 min in 2.5% copper sulfate); MST-16 (20 min in 5% copper sulfate); MST-18 (10 min in 5% copper sulfate); and MST-19 (30 min in 5% copper sulfate).

In the antagonism studies, the symbiont isolates did not inhibit growth of any *Phytophthora* species or *Pythium vexans.*

The following fungi or groups were separated on the basis of similarities and dissimilarities in various cultural and chemical characteristics.

Thelephora terrestris 1, 2, and V-9037—Radial growth after 25 days at 25 °C in darkness averaged 18 mm on Hagem, 22 mm on MMN, and 12 mm on PDA media. Both aerial and submerged mycelia were evident on Hagem agar. The aerial mycelium was white at the center, and the edge of the colony was somewhat darker and irregular in shape. No droplets were produced on the colony surface. Reverse center of the colony was cartridge buff merging into white. Reaction of mycelium to 5% KOH or 10% NH_4OH was not detected. The isolates caused a 5-mm zone of inhibition to *Pythium aphanidermatum.* Hyphal diameters averaged 3.3 μ (range 2.5 to 4.5 μ) on MMN agar. About 75 to 90% of the septa on main and lateral branches had typical clamp connections. Hyphal swelling and anastomosis were absent (Fig. 1A).

Thelephora terrestris S-142—Radial growth after 25 days at 25 °C in darkness averaged 6 mm on Hagem, 11 mm on MMN, and 4 mm on PDA media. The aerial mycelium was cottony on Hagem agar. The culture was drab-gray in the center merging into a white, irregular margin. Large, light-brown drops were found on the colony surface. Reverse center of the colony was cartridge buff merging into white. Mycelium did not react to 5% KOH or 10% NH_4OH. The isolate caused a 5-mm zone of inhibition to *Pythium aphanidermatum* and *P. irregulare.* Hyphal diameters averaged 4.1 μ (range 3 to 5 μ) on MMN agar, and about 80% of the septa had typical clamps (Fig. 1B).

Thelephora terrestris Z-13—Radial growth after 25 days at 25 °C in darkness averaged 13 mm on Hagem, 15 mm on MMN, and 5 mm on PDA media. The aerial mycelium was white at the center and tawny at irregular margins on Hagem agar. No droplets were found on the colony surface. Reverse center of the colony was buffy olive merging into a narrow zone of honey yellow bordered by pale white. Reaction of mycelium to 5% KOH or 10% NH_4OH was not detected. The isolate did not inhibit any pythiaceous fungus. Hyphal diameters averaged 4.0 μ (range 2.5 to 5.5 μ) on MMN agar. About 90% of the septa on main and lateral branches had clamp connections; half were typical clamps and half were knobby clamps. Hyphae were wavy without swellings or anastomosis (Fig. 1C).

Thelephora terrestris OKM-3838—Radial growth after 25 days at 25 °C in darkness averaged 12 mm on Hagem and 16 mm on MMN agar media. The aerial mycelium was sparse, was white, and had an irregular margin on Hagem agar. No droplets were found on the

colony surface. Reverse center of the colony was verona brown merging into a narrow zone of cinnamon buff bordered by a wide margin of pale white. Reaction of mycelium to 5% KOH or 10% NH_4OH was not detected. The isolate did not inhibit any pythiaceous fungus. Hyphal diameters averaged 3.4 μ (range 2.5 to 4.5 μ) on MMN agar, and about 90% of the septa on main and lateral branches had typical clamps. Hyphae were wavy with anastomosis but without swelling (Fig. 1D).

Group 1. Isolates MST-6, 7, 8, 9, 10, 11, and 13—Radial growth after 25 days at 25 °C in darkness averaged 18 mm on Hagem, 30 mm on MMN, and 18 mm on PDA media. The colony on Hagem agar was mostly submerged, with some sparse, pale-white mycelium. The aerial mycelium extended on the colony surface beyond the submerged mycelium. Light-brown droplets were present on the colony surface. Reverse center of the colony was cartridge buff to honey yellow merging into a large, pale-white, irregular margin. Reaction of mycelium to 5% KOH or 10% NH_4OH was not detected. The isolates caused a 5-mm zone of inhibition to *Pythium aphanidermatum*, *P. irregulare*, and *P. spinosum*. Hyphal diameters averaged 3.3 μ (range 2.0 to 5.0 μ) on MMN agar, and about 90% of the septa on main and lateral branches had half typical clamps and half knobby clamps. Hyphae were slightly wavy with a few terminal hyphal swellings and no anastomosis (Fig. 1E).

Group 2. MST-12—Radial growth after 25 days at 25 °C in darkness averaged 10 mm on Hagem, 12 mm on MMN, and 21 mm on PDA media. The colony on Hagem agar had abundant, cottony, aerial mycelium, white in the center and merging into an irregular margin of light olive-gray. Small, clear droplets were present on the colony surface. Reverse center of the colony was tawny olive merging into narrow margin of pale olive-gray. Drops of 5% KOH and 10% NH_4OH turned dark reddish brown within 1 min in the presence of mycelium. The isolate caused a 5-mm zone of inhibition to *Pythium aphanidermatum*, *P. irregulare*, *P. spinosum*. Hyphal diameters averaged 4.3 μ (range 3 to 6 μ) on MMN agar, and about 90% of the septa on main and lateral branches had large, typical clamps. Hyphae were wavy without swelling or anastomosis (Fig. 1F).

Group 3. MST-14—Radial growth after 25 days at 25 °C in darkness averaged 8 mm on Hagem, 13 mm on MMN, and 21 mm on PDA media. The colony on Hagem agar was medium with abundant, cottony to woolly, aerial mycelium of pale olive-gray. Reverse center of the colony was mouse gray, merging sharply into a wide zone of light grayish olive bordered by light mineral-gray at an irregular margin. A few clear droplets were found on the colony surface. Reaction of mycelium to 5% KOH or 10% NH_4OH was not detected. The isolate caused a 5-mm zone of inhibition to *Pythium aphanidermatum*, *P. irregulare*, and *P. spinosum*. Hyphal diameters averaged 2.9 μ (range 2.5 to 4.0 μ) on MMN agar, and about 90% of the septa on main and lateral branches had typical clamps. Intercalary hyphal swellings and anastomosis were present (Fig. 1G).

Group 4. MST-15—Radial growth after 25 days at 25 °C in darkness averaged 9 mm on Hagem, 13 mm on MMN, and 17 mm on PDA media. The colony on Hagem agar had abundant, woolly, aerial mycelium of pale gray. Reverse center of the colony was Saccardo's amber merging sharply into a narrow zone of cinnamon buff bordered by a white, even to slightly irregular margin. No droplets were found on the colony surface. Reaction of mycelium to 5% KOH or 10% NH_4OH was not detected. The isolate caused a 5-mm zone of inhibition to *Pythium aphanidermatum*, *P. irregulare*, and *P. spinosum*. Hyphal diameters averaged 3.8 μ (range 2.5 to 5.0 μ) on MMN agar, and about 90% of the septa on main and lateral branches had half typical clamps and half knobby clamps. Hyphae were slightly wavy with few, intercalary, hyphal swellings and no anastomosis (Fig. 1H).

Group 5. MST-16 and 19—Radial growth after 25 days at 25 °C in darkness averaged 5 mm on Hagem, 3 mm on MMN, and no growth on PDA media. The colony on Hagem agar had a small amount of white, aerial mycelium, and the submerged mycelium was russet. The reverse colony was entirely russet to mars brown with an irregular margin. No droplets were found on the colony surface. Reaction of mycelium to 5% KOH or 10% NH_4OH was not detected. The isolates caused a 5-mm zone of inhibition to *Pythium aphanidermatum* and *P. irregulare*. Hyphal diameters averaged 3.4 μ (range 2.5 to

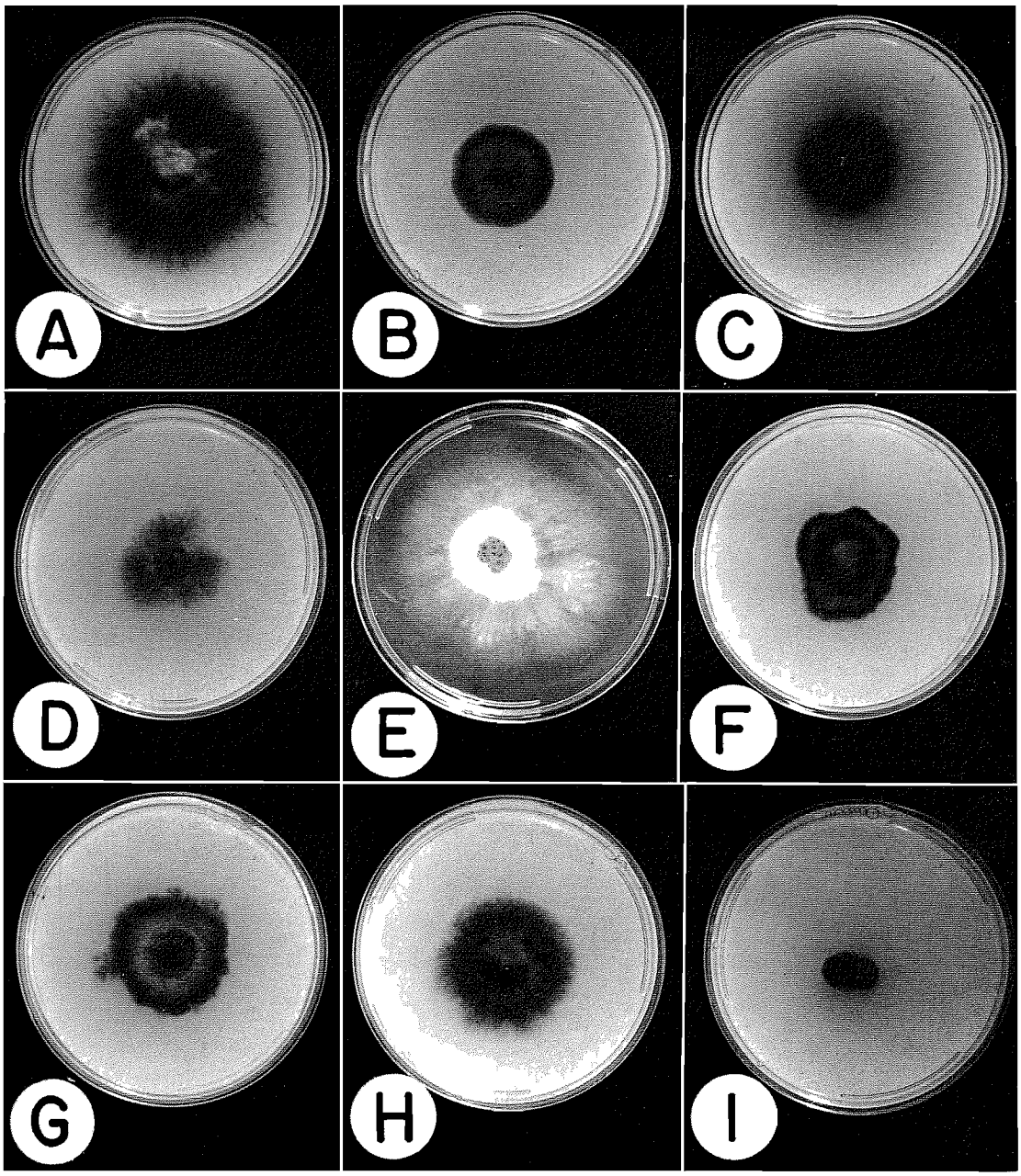

FIG. 1. Cultural groups of ectomycorrhizal fungi from shortleaf pine on modified Melin-Norkrans agar medium after 25 days at 25 °C. *Thelephora terrestris* 2 (A), S-142 (B), Z-13 (C), OKM-3838 (D), MST-6 (E), MST-12 (F), MST-14 (G), MST-15 (H), and MST-16 (I).

5.0 μ) on MMN agar, and about 90% of the septa on main and lateral branches had two-thirds typical clamps and one-third knobby clamps. Hyphae were slightly wavy with few, intercalary, hyphal swellings and no anastomosis (Fig. 1I).

Discussion

At least five, culturally different, ectomycorrhizal fungi of shortleaf pine colonized the fumigated soil within 9 months in the greenhouse. The first symbiont group, represented by seven isolates, is culturally and microscopically identical with *Thelephora terrestris* isolates 1, 2, and V-9037. The other groups, represented by one or two isolates each, do not resemble *T. terrestris* and are assumed to be different symbiont species.

In the southeastern United States, the rapid colonization of fumigated soil by *T. terrestris* and other ectomycorrhizal fungi, presumably by airborne basidiospores, assures good ectomycorrhizal development on roots of nursery seedlings. Good ectomycorrhizal development on roots of southern pines in the nursery is essential to seedling survival in the field (5).

The six isolates of *T. terrestris* examined in this study were all similar in respect to growth on various media and other characteristics. Each grew best on MMN. Growth was intermediate on Hagem agar and least or nonexistent on PDA. Media preference, colony color of both surface and undersurface mycelium, hyphal diameters, and type of clamp connection were valuable diagnostic characteristics for these isolates.

Antagonism of the isolates toward different pythiaceous fungi was consistent for each group, but only three *Pythium* species were sensitive. This feature has somewhat limited diagnostic value in these comparisons but may be valuable for comparisons of other ectomycorrhizal fungi encountered in subsequent research.

All surface sterilants except mercuric chloride and copper sulfate gave very poor direct isolation of symbionts from ectomycorrhizae in this study. Apparently, the latter two chemicals are selective: the group of isolates similar to *T. terrestris* was isolated with mercuric chloride, and the other groups were isolated with copper sulfate. It is interesting to consider the possible number of different species of ectomycorrhizal fungi that might have been recovered from these seedlings if better reisolation had been obtained.

1. DOMINIK, T. 1961. Studies on mycorrhizae (Studium o mikoryzie). Folia Forest. Polon. Ser. A, No. 5. pp. 3–160. Transl. Sci. Publ. Foreign Coop. Center Cent. Inst. Sci., Tech. and Econ. Inform. Warsaw, Poland. 1966.
2. FASSI, B., and A. FONTANA. 1966. Researches on ectotrophic mycorrhizae of *Pinus strobus* in nurseries. II. Mycorrhizae of *Thelephora terrestris* Ehrh. ex Fries, *Laccaria laccata* (Scop.) Berk., and *Hebeloma mesophaeum* Pers. ex Fries. Allionia (Turin), **12**: 47–53.
3. HACSKAYLO, E. 1965. *Thelephora terrestris* and mycorrhizae of Virginia pine. Forest Sci. **11**: 401–404.
4. HARLEY, J. L. 1959. The biology of mycorrhiza. Interscience Publishers, Inc., New York. p. 234.
5. JORGANSON, J. R., and E. SHOULDERS. 1967. Mycorrhizal root development vital to survival of slash pine nursery stock. U.S.D.A. Forest Serv. Tree Planters Notes, **18**: 7–11.
6. MARX, D. H. 1969. The influence of ectotrophic mycorrhizal fungi on the resistance of pine roots to pathogenic infections. I. Antagonism of mycorrhizal fungi to root pathogenic fungi and soil bacteria. Phytopathology, **59**: 153–163.
7. MARX, D. H., and W. C. BRYAN. 1969. Studies on ectomycorrhizae of pine in an electronically air-filtered, air-conditioned, plant-growth room. Can. J. Bot. **47**: 1903–1909.
8. MARX, D. H., and C. B. DAVEY. 1969. The influence of ectotrophic mycorrhizal fungi on the resistance of pine roots to pathogenic infections. IV. Resistance of naturally occurring mycorrhizae to infections by *Phytophthora cinnamomi*. Phytopathology, **59**: 559–565.
9. MODESS, O. 1941. Zur kenntinis der mykomhizabildner von kiefer und fichte. Symb. Bot. Upsal. **5**: 1–147.
10. PEYRONEL, B., B. FASSI, A. FONTANA, and J. M. TRAPPE. 1969. Terminology of mycorrhizae. Mycologia, **61**: 410–411.
11. SCHRAMM, J. R. 1966. Plant colonization studies on black wastes from anthracite mining in Pennsylvania. Trans. Amer. Phil. Soc. **56**(1): 1–194.
12. SHEMAKHANOVA, N. M. 1967. Mycotrophy of woody plants (Mikotrotiya drevesnykh porod). Acad. Sci. U.S.S.R., Inst. Microb. Israel Prog. Sci. Transl. Jerusalem. p. 329.
13. ZAK, B., and D. H. MARX. 1964. Isolation of mycorrhizal fungi from roots of individual slash pines. Forest Sci. **10**: 214–222.